Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector

Guido Favia*†, Irene Ricci*, Claudia Damiani*, Noura Raddadi‡, Elena Crotti‡, Massimo Marzorati‡, Aurora Rizzi‡, Roberta Urso‡, Lorenzo Brusetti‡, Sara Borin‡, Diego Mora‡, Patrizia Scuppa*, Luciano Pasqualini*, Emanuela Clementi§, Marco Genchi§, Silvia Corona§, Ilaria Negri¶, Giulio Grandi‖, Alberto Alma¶, Laura Kramer‖, Fulvio Esposito*, Claudio Bandi**, Luciano Sacchi§, and Daniele Daffonchio†‡

*Dipartimento di Medicina Sperimentale e Sanità Pubblica, Università degli Studi di Camerino, 62032 Camerino, Italy; Dipartimenti di ‡Scienze e Tecnologie Alimentari e Microbiologiche and **Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Università degli Studi di Milano, 20133 Milan, Italy; §Dipartimento di Biologia Animale, Università degli Studi di Pavia, 27100 Pavia, Italy; ¶Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Università degli Studi di Torino, 10095 Torino, Italy; and ‖Dipartimento di Produzioni Animali, Facoltà di Medicina Veterinaria, Università degli Studi di Parma, 43100 Parma, Italy

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Here, we show that an α-proteobacterium of the genus *Asaia* is stably associated with larvae and adults of *Anopheles stephensi*, an important mosquito vector of *Plasmodium vivax*, a main malaria agent in Asia. *Asaia* bacteria dominate mosquito-associated microbiota, as shown by *16S rRNA* gene abundance, quantitative PCR, transmission electron microscopy and *in situ*-hybridization of *16S rRNA* genes. In adult mosquitoes, *Asaia* sp. is present in high population density in the female gut and in the male reproductive tract. *Asaia* sp. from *An. stephensi* has been cultured in cell-free media and then transformed with foreign DNA. A green fluorescent protein-tagged *Asaia* sp. strain effectively lodged in the female gut and salivary glands, sites that are crucial for *Plasmodium* sp. development and transmission. The larval gut and the male reproductive system were also colonized by the transformed *Asaia* sp. strain. As an efficient inducible colonizer of mosquitoes that transmit *Plasmodium* sp., *Asaia* sp. may be a candidate for malaria control.

malaria | symbiotic control | insect vector

One of the major objectives of malaria control programs is interference with parasite transmission by mosquito vectors (1). Mosquito genetic transformation has been developed for *Anopheles gambiae* (2) and *Anopheles stephensi* (3), the main malaria vectors in Africa and Asia, respectively, and transgenic mosquitoes have been developed that are impaired in parasite transmission (4). However, genetic manipulation tends to reduce mosquito fitness (5). There is current interest in the use of microorganisms as biological control agents of vector-borne diseases (6–8). Microorganisms associated with vectors could exert a direct pathogenic effect on the host (9), interfere with its reproduction (10, 11), or reduce vector competence (12, 13). Furthermore, the use of genetically modified bacteria to deliver antiparasite molecules has several advantages over the use of genetically modified vectors (14). Very little is currently known about mosquito-associated microbiota (14–16). Here, we show that bacteria of the genus *Asaia* are stably associated with *An. stephensi*, an Asian malarial mosquito vector. *Asaia* can be cultivated and genetically manipulated and can recolonize the insect host. As an efficient inducible colonizer of mosquitoes that transmit *Plasmodium* sp., *Asaia* sp. may be a candidate for malaria control.

Results and Discussion

***16S rRNA* Gene Libraries from *Anopheles* spp. and *Asaia* sp. PCR Screening.** Bacterial diversity associated with *An. stephensi* was initially explored by establishing three *16S rRNA* gene libraries from total DNA of the abdomens of three laboratory-bred individuals. Analysis of the *16S rRNA* gene sequence revealed that the libraries were dominated by sequences related to the genus *Asaia* [90% of the clones examined; see supporting information (SI) Table 2], an α-proteobacterium strictly related to acetic acid bacteria (17, 18). The obtained sequence showed >99% nucleotide identity with those of *Asaia bogorensis* and *Asaia siamensis*, two species previously isolated from tropical flowers (17, 18). Other clones were related to different α-proteobacteria (*Acetobacter*, *Gluconobacter*, and *Sphingomonas*; see SI Table 2). By using a *16S rRNA*-based *Asaia*-specific PCR, amplicons were found in both males and females of all of the >300 individuals of *An. stephensi* tested derived from at least 10 different breeding batches (SI Table 3). The identity of the amplicons was confirmed by sequencing. PCR analysis showed that *Asaia* DNA is present in eggs, pupae, and different larval stages, as well as in various mosquito organs, including gut, salivary glands, ovaries, and testes. *Asaia* DNA was also found in all of the 60 field-collected individuals of *Anopheles maculipennis* captured during three different periods (June 2005, October 2005, and June 2006) and in their F1 obtained in the laboratory (SI Table 3). *Asaia* represented 20% of the clones in *16S rRNA* gene libraries (SI Table 4). *Asaia* was sporadically (≈5% of the clones) found in *16S rRNA* gene libraries from the total DNA of *An. gambiae* individuals collected in Burkina Faso (SI Tables 3 and 5). Interestingly, it has recently been reported that interference with the innate immune system of *An. gambiae* by transient silencing of *AgDscam* determines proliferation of *A. bogorensis* in mosquito hemolymph (19).

Diversity of bacteria within the *16S rRNA* gene libraries was rather low, with relatively few phylotypes. Low bacterial diversity in *Anopheles* species by *16S rRNA* gene sequencing has been

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Abbreviations: Gfp, green fluorescent protein; ISH, *in situ* hybridization; TEM, transmission electron microscopy.

Data deposition: The *16S rRNA* gene sequences of isolates reported in this paper have been deposited in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GeneBank databases (accession nos. AM404260, AM404261, and AM404262).

†To whom correspondence may be addressed. E-mail: guido.favia@unicam.it or daniele.daffonchio@unimi.it.

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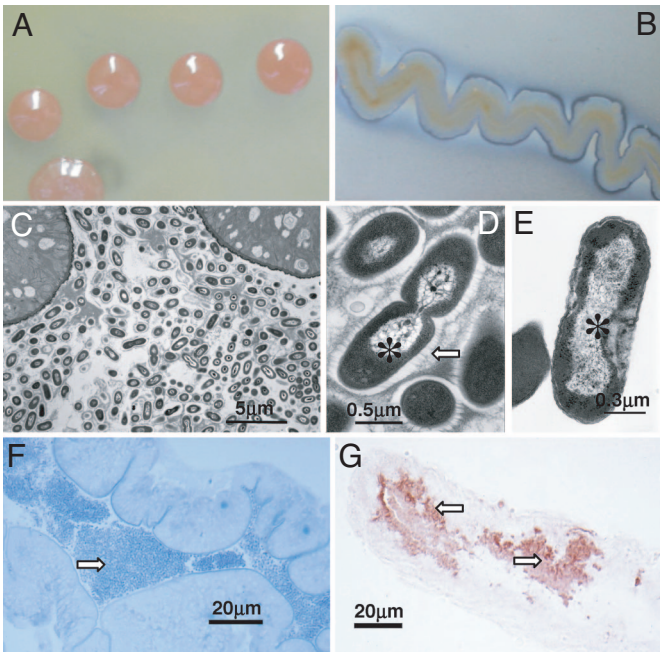

Fig. 1. Images of *Asaia* sp. from *An. stephensi*. (*A*) Colonies of *Asaia* sp. on $CaCO_3$-agarized medium, showing a characteristic pink color. (*B*) Haloes of carbonate solubilization due to acidification. (*C*) TEM micrograph of an *An. stephensi* adult midgut full of bacteria that probably belongs to genus *Asaia*. (*D* and *E*) Magnification of bacterial cells in the midgut (*D*), whose morphology resembles that of *Asaia* sp. in pure culture (*E*). Filamentous structures in the nucleoid region (asterisks), and an extracellular matrix with a fibrillar nature (arrow) can be noted. Cells of *Asaia* sp. in pure culture do not show the extracellular matrix observed in the mosquito midgut. (*F*) The midgut lumen of *An. stephensi* is full of bacterial cells (arrow). (*G*) ISH with an *Asaia*-specific probe showing a high density of the bacterium in the midgut lumen (arrows).

reported, with six, two, and one bacterial species in *An. arabiensis*, *An. gambiae* sensu stricto, and *An. funestus*, respectively (16). We detected few operational taxonomic units within the γ-proteobacteria that were detected in other studies by *16S rRNA* gene sequencing (16) and bacterial isolation (14, 16). This difference may be due to the different primer set used and emphasizes that the use of multiple primer sets in molecular microbial ecology widens the view of the actual diversity residing in a system (20).

***Asaia* Cultivation and Real-Time PCR Analysis.** *Asaia* was isolated from *An. stephensi* according to the method reported for isolation from tropical flowers (17, 18). A preenrichment step in liquid medium at pH 3.5, followed by plating in carbonate-rich medium, resulted in the isolation of single, pink colonies capable of dissolving carbonate in the medium and generating dissolution haloes (Fig. 1 *A* and *B*). Colonies isolated from *An. stephensi* and *An. maculipennis* were confirmed to be *Asaia* sp. by *16S rRNA* gene sequencing, with 99.6% and 99.8% nucleotide identity with *A. bogorensis* and *A. siamensis*, respectively (17, 18) (SI Fig. 4). *Asaia* sp. was abundant in the mosquito body, with bacterial counts of up to 9.8×10^5 colony forming units (CFU) per female and 9.8×10^4 CFU per male individuals. To evaluate the relative abundance of *Asaia* sp. in different organs of *An. stephensi*, we measured *Asaia* to total bacteria *16S rRNA* gene Asaia to total bacteria copy ratio (ABR) with quantitative real-time PCR, by determining the *Asaia* sp. and total bacteria *16S rRNA* gene copies in the gut, salivary glands, and female reproductive system (Table 1). *Asaia* sp. *16S rRNA* gene copies constituted a mean of 41%, 25%, and 20% of the total *16S rRNA* gene copies of the bacterial population in the gut, salivary glands, and female reproductive system, respectively, showing that this acetic bacterium represents the dominant bacterium in the population of *An. stephensi* examined in this study, particularly in the gut. Interestingly, high numbers of *Asaia* were also found in both the salivary glands, which have rarely been reported to be colonized by bacteria in other insects (21), and in the female reproductive system, where vertically transmitted endosymbionts are typically found.

Transmission Electron Microscopy (TEM) and *in Situ* Hybridization (ISH) Analysis of *Asaia* in Mosquito. Several body parts of female *An. stephensi* were examined by TEM, and the morphology of observed bacteria was compared with that of cultured *Asaia*. No bacteria were detected in the egg cell cytoplasm, whereas the midgut lumen contained large bacterial cell clusters with the typical Gram-negative architecture with a signature filamentous appearance of the nucleoid region similar to cultured *Asaia* (Fig. 1 *C–E*). The bacteria in the insect midgut were embedded within an extracellular matrix (Fig. 1*D*). The identity of the bacteria in *An. stephensi* midgut (Fig. 1*F*) was confirmed by ISH with *Asaia*-specific probes that indicated a high concentration of cells within the mucous substance lining the midgut epithelium (Fig. 1*G*). ISH signals with *Asaia*-specific probes were analogous to those observed by using bacterial probe EUB338, whereas no signals were observed in tissues treated with RNase or in the absence of the probe.

Mosquito Recolonization by *Asaia* Expressing Green Fluorescent Protein (Gfp). The overall data indicated that *Asaia* bacteria are dominant in, and stably associated with, the midgut of *An. stephensi* and hence could be an interesting vector of antiparasite molecules in mosquitoes. The transformability of *Asaia* from *An. stephensi* was verified by electroporating *Asaia* sp. strain SF2.1 with plasmids pHM2 and pHM3, two replicative plasmids in the genera *Acetobacter* and *Gluconobacter* closely related to the genus *Asaia* (22). The plasmids transformed strain SF2.1 with an efficiency of 4.7 10^5 cells·μg^{-1} DNA. Plasmid pHM2 conferred both kanamycin resistance and the blue colony phenotype to the strain when the cells were grown in the presence of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside, whereas plasmid pHM3 conferred resistance to the antibiotic but not blue colony phenotype. We cloned a Gfp cassette in plasmid pHM2 to label

Table 1. *Asaia* sp. and bacteria *16S rRNA* gene copies and *Asaia* to bacteria *16S rRNA* gene copy ratio (ABR) in different organs of *An. stephensi*

Organ	No. of individuals*	Range of *Asaia 16S rRNA* gene copies†	Range of bacterial *16S rRNA* gene copies†	ABR‡	ABR range
Gut	22	1.3×10^4 to 8.7×10^7	1.6×10^5 to 9.5×10^7	0.41 ± 0.28	0.03–0.91
Salivary glands	17	5.3×10^2 to 4.0×10^6	1.2×10^5 to 6.7×10^6	0.25 ± 0.25	0.004–0.62
Female reproductive system	17	7.5×10^1 to 3.4×10^6	4.0×10^4 to 4.9×10^6	0.20 ± 0.27	0.001–0.78

*Each individual was separately analyzed by triplicate real-time PCR.
†Minimum and maximum amounts of *16S rRNA* copies detected.
‡ABR, *Asaia* to total bacterial *16S rRNA* gene copy ratio. Mean ABR ± SD of the individuals reported in column 2 are given.

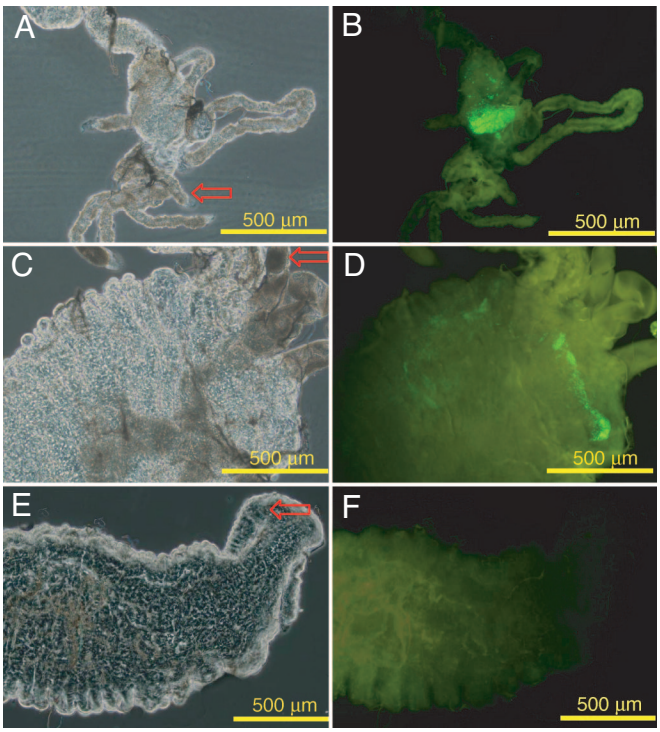

Fig. 2. Recolonization of the adult *An. stephensi* gut by a Gfp-tagged *Asaia*. Phase contrast (*A* and *C*) and fluorescence (*B* and *D*) microscope images of *Asaia* sp. strain SF2.1(Gfp), recolonizing *An. stephensi* midgut. Male (*A* and *B*) and female (*C* and *D*) terminal portions of the midgut. Malpighian tubules are visible (red arrows). (*E* and *F*) Midgut terminal portion of a female fed with sucrose solution without *Asaia* sp. strain SF2.1(Gfp). (*G* and *H*) Laser scanning confocal microscope images of adult mosquito guts, showing high concentrations of strain SF2.1(Gfp) microcolonies.

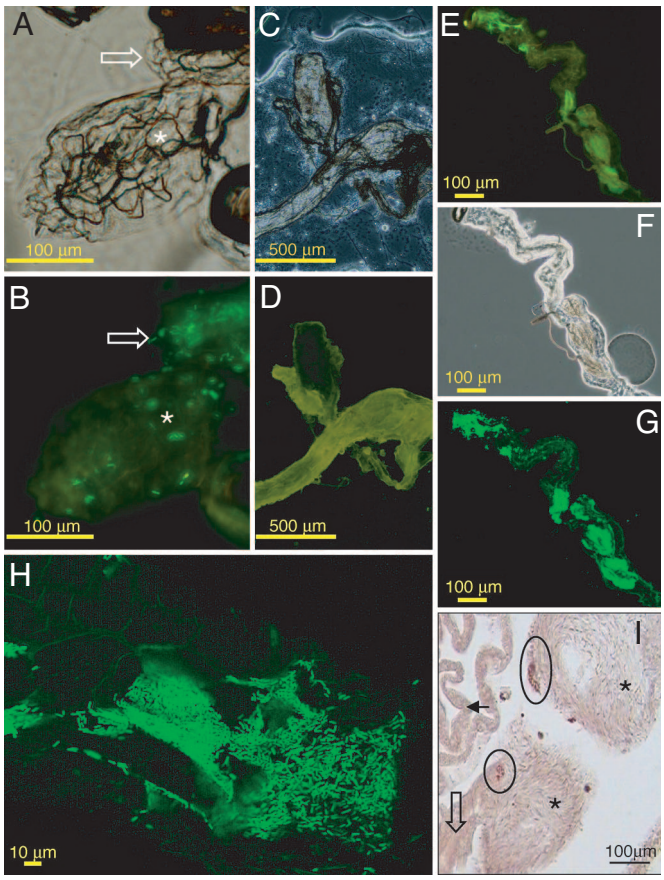

Fig. 3. Colonization of male gonads of adult *An. stephensi* by *Asaia* sp. A large number of *Asaia* cells was found in the reproductive organs of adult, male *An. stephensi*. (*A–D*) Reproductive organs of an *An. stephensi* adult male fed (*A* and *B*) or not fed (*C* and *D*) with a sucrose-containing solution in which *Asaia* sp. SF2.1(Gfp) cells were suspended. A testis (asterisk) and initial portion of a gonoduct (arrow) are visible by phase contrast (*A* and *C*) and fluorescence (*B* and *D*) microscopy. Fluorescent *Asaia* sp. SF2.1(Gfp) cells are visible in the testis and in the gonoducts. (*E–G*) Male gonoduct, after colonization by *Asaia* sp. strain SF2.1(Gfp), visualized by fluorescent (*E*), phase contrast (*F*), and laser scanning confocal microscopy (*G*). The images indicate a high concentration of *Asaia* sp. is in the gonoduct. (*H*) Higher magnification of a microcolony of *Asaia* sp. strain SF2.1(Gfp) in the male gonoduct. (*I*) ISH showing cell clusters of *Asaia* sp. cells (circles) colonizing the male reproductive organs. Asterisks indicate spermatic bundles. A sperm duct (open black arrow) is visible. Intestine is indicated by a black arrow. The specimen pictured was taken from a male not exposed to strain SF2.1(Gfp).

Asaia with an easy-to-follow optical marker for visually tracking the bacterium in the body of *An. stephensi*. The resulting plasmid pHM2-Gfp was used to transform strain SF2.1. We obtained strain SF2.1(Gfp) that was then used in recolonization experiments of *An. stephensi* adults and larvae.

The SF2.1(Gfp) strain recolonized *An. stephensi* adults fed with sucrose or blood-containing solutions in which the strain was resuspended. Fluorescent and laser-scanning confocal microscopy showed that the bacterium efficiently colonized the gut (Fig. 2), salivary glands (SI Fig. 5) and male reproductive system (Fig. 3*A–H*). We examined a total of 140 insect adults from three cages fed with *Asaia* sp. strain SF2.1(Gfp). Most of the individuals (72%) showed fluorescent cells and microcolonies in the body. Because we do not know whether all of the individuals actually fed on the bacterial suspension, the lack of *Asaia* from some individuals, in the light of the high percentage of positives, could be explained with the lack of a meal. Fluorescent *Asaia* cells and microcolonies were observed in 65%, 32%, and 58% of the guts (91 individuals examined), salivary glands (59 females examined), and male reproductive systems (55 individuals examined). Colonization of the mosquito body by *Asaia* sp. strain SF2.1(Gfp) was stably maintained for the entire life span of the insect. Fluorescent cells and microcolonies were detected in all of the previously mentioned body parts (gut, salivary gland, and male reproductive system) of adult mosquitoes (10 individuals) up to 25 days, before insect death. Fluorescent cells and microcolonies were established in the gut after 24 h when mosquitoes were fed with blood and after 48 h when fed with sucrose-containing solutions. This difference in the timing of microcolony establishment could be due to the fact that sugar goes to the crop instead of mosquito midgut, determining a delay in dispersion to other portions of the gut. The consequent higher number of ingested bacterial cells likely allowed a more rapid colonization of the gut with the fluorescent *Asaia*. Strain SF2.1(Gfp) was capable of recolonizing salivary glands in a relatively short time (48 h) after ingestion of the sugar meal. Strain SF2.1(Gfp) was observed in the male reproductive system 48 h after the exposure of male mosquitoes to the sugar solution. Bacteria massively colonized testes, and, in particular, gonoducts (Fig. 3 *A–H*), demonstrating that the male reproductive system is a further subniche for *Asaia* in *An. stephensi*. Interestingly, large fluorescent microcolonies were observed within male gonoducts (Fig. 3*H*), indicating bacterial growth. Signals obtained by ISH with *Asaia*-specific probes also identified *Asaia* sp. in the spermatic bundles of *An. stephensi* males that were not exposed to strain SF2.1(Gfp) (Fig. 3*I*). A large number of bacterial cells resembling *Asaia* sp. was also observed by TEM in gonoducts of males not colonized by strain SF2.1(Gfp). These cells showed the typical morphology of *Asaia* sp. with signature

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filamentous structures in the nucleoid region and, like cultured *Asaia* cells (Fig. 1*E*), did not present the extracellular matrix that was observed in the mosquito midgut (Fig. 1*D*).

Second and third instar larvae of *An. stephensi* were also fed with strain SF2.1(Gfp) that was resuspended in the larvae breeding water. Colonization of the larval gut was observed at 24 h after exposure. We examined a total of 24 larvae (12 each for L2 and L3 stages). Seven L2 and five L3 larvae showed massive colonization in the gut by large fluorescent microcolonies.

The observation of *Asaia* in the male gonoduct suggested that transmission of *Asaia* from male to female may occur during mating. We thus caged 16 virgin females with 24 males previously fed with a cell suspension of strain SF2.1(Gfp). Eight of the 16 females showed fluorescent *Asaia* cells and microcolonies in the spermatheca and the gut. It is possible that the percentage of *Asaia*-positive females could be biased by a low mating rate due to a nonoptimal male-to-female ratio in the cage. Finally, 16 females of *An. stephensi* previously fed with strain SF2.1(Gfp) were allowed to have a blood meal and lay eggs. Thirty-two of the hatched larvae were then examined (eight individuals for each larval stage from L1 to L4) and 19 of these showed a massive colonization by fluorescent cells and microcolonies in the gut, indicating that the bacterium is transmitted to the offspring. This result is congruent with PCR data showing the presence of *Asaia* sp. DNA in preadult stages of *An. stephensi*. There is thus an overall consistency of results indicating that colonization of mosquitoes occurs early during their development. It is reasonable to assume that infection of mosquito larvae occurs by acquisition of *Asaia* sp. from the environment, if we consider, for example, the results reported above on the colonization of larvae by *Asaia* strain SF2.1(Gfp) released in the breeding water. However, the fluorescent *Asaia* cells that were detected in larvae generated by females fed on SF2.1(Gfp) strain indicate that *Asaia* is also transmitted from mother to offspring. Understanding whether this transmission is direct (e.g., based on some forms of transovarial transmission or egg smearing) or indirect (e.g., adult mosquitoes contaminate the environment during egg laying, and bacteria then infect the progeny) still remain to be determined.

Perspectives. Flowers and plant-derived materials have been reported as the natural habitats of *Asaia* spp. (17, 18, 23). Strains of the genus *Asaia* have also been isolated in bottled fruit-flavored drinks (24), and *A. bogorensis* was recently isolated from the blood of an i.v. drug user in Finland (25). Based on the results reported here, mosquitoes of the genus *Anopheles* could represent another environmental niche for *Asaia* where it lives at a particularly high density in the gut. This acetic acid bacterium could be taken up by mosquitoes from their environment, i.e., from water during the larval stages or from flowers during the first sugar meals as an adult. Thereafter, particular physiological and metabolic requirements would allow infection of the insect body. It would be interesting to study the environmental sources of infection of the mosquito body. Bacteria of the genus *Asaia* have not been previously reported as associated with any other entomological system, except with *Scaphoideus titanus* (Hemiptera: Cicadellidae), the vector of Flavescence Dorée in grapevines (8). The identification of bacteria of the genus *Asaia* in insect species of different orders like Diptera and Hemiptera suggests that they may be widespread symbionts of insects. The presence of *Asaia* in the three *Anopheles* species that we have examined and its very high prevalence in different developmental stages of *An. stephensi* and *An. maculipennis* indicate that *Asaia* may play an important role in the biology of the host that will be investigated in future studies. Here, we have shown that *Asaia* is capable of efficiently crossing body barriers and colonizing different organs in a short time, like guts and salivary glands, both important sites of *Plasmodium* parasites' life cycle. The ability of *Asaia* sp. to recolonize the mosquitoes was also confirmed by the capacity of the bacterium to grow quickly in several different body parts, as shown by the formation of microcolonies (Fig. 3*H*) and the identification of cells undergoing division (see for example Fig. 1*D*). The high level of mosquito colonization reached by *Asaia* sp. after feeding indicates that horizontal transmission by the oral route can efficiently lead to the infection of the mosquito body and could possibly be exploited in the field for insect colonization.

Vertical transmission to the progeny is commonly observed in insects for intracellular bacterial symbionts (26), whereas extracellular midgut bacteria are generally regarded as opportunistic microorganisms acquired from the environment (14). However, a clear-cut distinction between environmental acquisition and vertical transmission of symbionts is not always easy to establish, such as in the case of microorganisms living in the gut of wood-feeding cockroaches and termites (27). Our results show that the *Anopheles–Asaia* symbiotic system might represents such a borderline situation, where acquisition from the environment is likely the most common source of infection for both preadult and adult stages, but where transmission from mother to offspring might also occur, leading to an efficient exploitation of the insect niche by this environmental bacterium. The efficiency by which *Asaia* might exploit the insect niche is also highlighted by the capacity demonstrated by strain SF2.1(Gfp) of colonizing different body parts and by its transmission from male to female during mating, as it has recently been shown for beneficial secondary symbionts in aphids (28).

Easy acquisition by both mosquito adults and larvae, culturability, cryogenic preservability, and the easy transformability of *Asaia* spp. make it a candidate for paratransgenic control (29) of malaria (14), through the production of anti-*Plasmodium* molecules (30–34) or anti-mosquito factors (15) by engineered bacterial strains (30).

Materials and Methods

Mosquitoes. *An. stephensi* samples came from a colony reared since 1988 in the insectary at the University of Camerino. *An. maculipennis* specimens were sampled in June and October 2005 and June 2006, inside a cowshed of a small farm close to Orte, in central Italy. *An. gambiae* specimens were field-collected in various villages of Burkina Faso (West Africa) in the period 2002–2004.

DNA-Based Analysis of the Mosquitoes' Microflora. DNA extraction from whole insects and organs/tissues was performed as described (35). Extracted DNA was used as template in PCRs with universal *16S rRNA* bacterial primers 27F 5′-TCGACATCGTTTACGGCGTG-3′ and 805R 5′-AGAGTTTGATCCTGGCTCAG-3′ and conditions reported in *SI Materials and Methods*. For *Asaia*-specific PCR, primers sets 20F–1500R, 520F–520R, and 920F–920R (17) and primers Asafor (5′-GCGCGTAGGCGGTTTACAC-3′) and Asarev (5′-AGCGTCAGTAATGAGCCAGGTT-3′) have been used following conditions described elsewhere (17) (see also *SI Materials and Methods*). Sequences of the purified amplicons were analyzed by using BLASTn (www.ncbi.nlm.nih.gov/blast/) and RDPII (http://rdp.cme.msu.edu), and comparative analysis was performed by ClustalX v.1.83 (36) and Treecon v.1.3b software (Van de Peer, University of Konstanz, Konstanz, Germany). Bacteria *16S rRNA* gene copies in the total DNA extracted from insect organs were determined by quantitative real-time-PCR in a I-cycler thermal cycler (Bio-Rad, Hercules, CA) by using primers 357F (5′-CTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) (37). For *Asaia*, primers Asafor and Asarev were used. The reactions were performed with Brilliant Sybr green qPCR Master Mix (Stratagene, La Jolla, CA).

TEM and ISH. Adults of *An. stephensi* were dissected in saline. Semithin sections for light microscopy and thin sections for TEM were prepared and examined as described (8). ISH was performed on paraffin-embedded sections of mosquito organs as described (38). Formamide concentration was adjusted at 30%, and hybridization temperature was set at 46°C. Probe Eub338 was used as a bacterial positive control, and probes Asaia1 (5′-AGCACCAGTTTCCCGATGTTAT-3′) and Asaia2 (5′-GAAATACCCATCTCTGGATA-3′), designed to target the *16S rRNA* were used for specific detection of *Asaia* cells. All of the probes were 5′-labeled with digoxigenin, and anti-digoxigenin/horseradish peroxidase antibodies were used as secondary antibodies. Staining was performed with 3-amino-9-ethylcarbazole and observation was with a light microscope (see also *SI Materials and Methods*).

***Asaia* Isolation, Identification, Count in Mosquito Individuals, and Transformation.** *Asaia* was isolated by a preenrichment step in liquid medium (pH 3.5) and hence plated in agarized medium as described (17, 18). *Asaia* colonies were tentatively identified by colony morphology and the formation of carbonate dissolution haloes in agar plates. Identification was confirmed by *16S rRNA* gene sequencing. *Asaia* cells in the whole insect were counted in 96-well microtiter plates by the most-probable number method in liquid medium, followed by plating on calcium carbonate-containing medium (17, 18) from the growth-positive wells. Colony identity was confirmed by *16S rRNA* gene sequencing of randomly picked colonies. *Asaia* sp. cells were electroporated with plasmids pHM2 and pHM3 (21) as reported in *SI Materials and Methods*. A *gfp* gene cassette was cloned in pHM2 plasmid as reported in *SI Materials and Methods* and electroporated in *Asaia* sp. strain SF2.1 isolated from an *An. stephensi* female. Successful transformants were confirmed by fluorescence microscopy, plasmid analysis, and PCR of the *gfp* gene cassette as reported in *SI Materials and Methods*. A Gfp-tagged transformant, designated as strain SF2.1(Gfp), was hence selected for mosquito recolonization experiments.

Mosquito Recolonization by *Asaia* sp. SF2.1(Gfp). *Asaia* sp. SF2.1(Gfp) was grown 24 h at 30°C in GLY medium (*SI Materials and Methods*). Cells were harvested by centrifugation, washed three times in 0.9% (wt/vol) NaCl and adjusted to 10^3 or 10^8 cells per ml^{-1} in 30 ml of H_2O/5% (wt/vol) sucrose solution or in 1 ml of human blood to feed adult mosquitoes. For monitoring long-term colonization of *An. stephensi*, the suspension containing 10^3 cells per ml^{-1} was supplemented with 50 $\mu g \cdot ml^{-1}$ of kanamycin to avoid the loss of plasmid from bacterial cells. Cages were prepared to feed *An. stephensi* adult with 2×10^8 or 4×10^8 cells per ml^{-1} of *Asaia* sp. strain SF2.1(Gfp), respectively. After 24, 48, 72, and 96 h of exposure to the feeding solutions containing strain SF2.1(Gfp), mosquitoes were dissected in PBS. One additional cage was fed with 4×10^3 cells per ml^{-1} of *Asaia* sp. strain SF2.1(Gfp), resuspended in 5% (wt/vol) sucrose solution. After 2 days feeding, the cotton swab containing strain SF2.1(Gfp) was removed and replaced with a new sterile 5% (wt/vol) sucrose solution supplemented with kanamycin. Mosquitoes fed with 4×10^3 cells per ml^{-1} of *Asaia* sp. strain SF2.1(Gfp) were sampled every 2–3 days up to 25 days after initial exposure to the bacterium. To feed 2nd and 3rd instar larvae, 10^8 cells per ml^{-1} were released in 40-ml batches of breeding water (each containing 60 larvae). The mosquitoes and the larvae were maintained with their usual diet. Guts, salivary glands, and reproductive organs were examined with a IX71 fluorescent microscope (Olympus, Melville, NY), and a MRC600 laser scanning confocal microscope (Bio-Rad). All tissues were fixed with 4% paraformaldeyde for 10 min at 4°C, with the exception of salivary glands. The slides were then mounted in glycerol–PBS for analysis. Whole larvae were directly observed without dissection after several washings in sterile distilled water.

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1. Atkinson PW, Michel K (2002) *Genesis* 32:42–48.
2. Grossman GL, Rafferty CS, Clayton JR, Stevens TK, Mukabayire O, Benedict MQ (2001) *Insect Mol Biol* 10:597–604.
3. Catteruccia F, Nolan T, Loukeris TG, Blass C, Savakis C, Kafatos FC, Crisanti A (2000) *Nature* 405:959–962.
4. Ito J, Ghosh A, Moreira AL, Wimmer EA, Jacobs-Lorena M (2002) *Nature* 417:452–455.
5. Catteruccia F, Godfray HC, Crisanti A (2003) *Science* 299:1225–1227.
6. Beard CB, Durvasula RV, Richards FF (1998) *Emerg Infect Dis* 4:581–591.
7. Beard CB, Cordon-Rosales C, Durvasula RV (2002) *Annu Rev Entomol* 47:123–141.
8. Marzorati M, Alma A, Sacchi L, Pajoro M, Palermo S, Brusetti L, Raddadi N, Balloi A, Tedeschi R, Clementi E, *et al.* (2006) *Appl Environ Microbiol* 72:1467–1475.
9. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Microbiol Mol Biol Rev* 62:775–806.
10. Zabalou S, Riegler M, Theodorakopoulou M, Stauffer C, Savakis C, Bourtzis K (2004) *Proc Natl Acad Sci USA* 101:15042–15045.
11. Zchori-Fein E, Gottlieb Y, Kelly SE, Brown JK, Wilson JM, Karr TL, Hunter MS (2001) *Proc Natl Acad Sci USA* 98:12555–12560.
12. Beard CB, Dotson EM, Pennington PM, Eichler S, Cordon-Rosales C, Durvasula RV (2001) *Int J Parasitol* 31:621–627.
13. Baldridge GD, Burkhardt NY, Simser JA, Kurtti TJ, Munderloh UG (2004) *Appl Environ Microbiol* 70:6628–6636.
14. Riehle MA, Jacobs-Lorena M (2005) *Insect Biochem Mol Biol* 35:699–707.
15. Khampang P, Chungjatupornchai W, Luxananil P, Panyim S (1999) *Appl Microbiol Biotechnol* 51:79–84.
16. Lindh JM, Terenius O, Faye I (2005) *Appl Environ Microbiol* 71:7217–7223.
17. Yamada Y, Katsura K, Kawasaki H, Widyastuti Y, Saono S, Seki T, Uchimura T, Komagata K (2000) *Int J Syst Evol Microbiol* 50:823–829.
18. Katsura K, Kawasaki H, Potacharoen W, Saono S, Seki T, Yamada Y, Uchimura T, Komagata K (2001) *Int J Syst Evol Microbiol* 51:559–563.
19. Dong Y, Taylor HE, Dimopoulos G (2006) *PLoS Biol* 4:e229.
20. Ben-Dov E, Shapiro OH, Siboni N, Kushmaro A (2006) *Appl Environ Microbiol* 72:6902–6906.
21. Cheng Q, Aksoy S (1999) *Insect Mol Biol* 8:125–132.
22. Mostafa HE, Heller KJ, Geis A (2002) *Appl Environ Microbiol* 68:2619–2623.
23. Bae S, Fleet GH, Heard GM (2006) *J Appl Microbiol* 100:712–727.
24. Moore JA, McCalmont M, Xu J, Millar BC, Heaney N (2002) *Appl Environ Microbiol* 68:4130–4131.
25. Tuuminen T, Heinäsmäki T, Kerttula T (2006) *J Clin Microbiol* 44:3048–3050.
26. Moran NA (2006) *Curr Biol* 16:R866–871.
27. Nalepa CA, Bignell DE, Bandi C (2001) *Insect Socieaux* 48:194–201.
28. Moran NA, Dunbar HE (2006) *Proc Natl Acad Sci USA* 103:12803–12806.
29. Bextine B, Lauzon C, Potter S, Lampe D, Miller TA (2004) *Curr Microbiol* 48:327–331.
30. Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, *et al.* (2006) *Nature* 440:940–943.
31. Pidiyar VJ, Jangid K, Patole MS, Shouche YS (2004) *Am J Trop Med Hyg* 70:597–603.
32. Aguilar R, Jedlicka AE, Mintz M, Mahairaki V, Scott AL, Dimopoulos G (2005) *Insect Biochem Mol Biol* 35:709–719.
33. Mourya DT, Gokhale MD, Pidiyar V, Barde PV, Patole M, Mishra AC, Shouche Y (2002) *Acta Virol* 46:257–260.
34. Pumpuni CB, Beier MS, Nataro JP, Guers LD Davis JR (1993) *Exp Parasitol* 77:195–199.
35. Favia G, Dimopoulos G, della Torre A, Toure YT, Coluzzi M, Louis C (1994) *Proc Natl Acad Sci USA* 91:10315–10319.
36. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) *Nucleic Acids Res* 25:4876–4878.
37. Muyzer G, de Waal EC, Uitterlinden AG (1993) *Appl Environ Microbiol* 59:695–700.
38. Beninati T, Lo N, Sacchi L, Genchi C, Noda H, Bandi C (2004) *Appl Environ Microbiol* 70:2596–2602.